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The Type I Interferon Signaling Pathway Is a Target for Glucocorticoid Inhibition[▽]

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Type I interferon (IFN) is essential for host defenses against viruses; however, dysregulated IFN signaling is causally linked to autoimmunity, particularly systemic lupus erythematosus. Autoimmune disease treatments rely on glucocorticoids (GCs), which act via the GC receptor (GR) to repress proinflammatory cytokine gene transcription. Conversely, cytokine signaling through cognate Jak/STAT pathways is reportedly unaffected or even stimulated by GR. Unexpectedly, we found that GR dramatically inhibited IFN-stimulated gene (ISG) expression in macrophages. The target of inhibition, the heterotrimeric STAT1-STAT2-IRF9 (ISGF3) transcription complex, utilized the GR cofactor GRIP1/TIF2 as a coactivator. Consequently, GRIP1 knockdown, genetic ablation, or depletion by GC-activated GR attenuated ISGF3 promoter occupancy, preinitiation complex assembly, and ISG expression. Furthermore, this regulatory loop was restricted to cell types such as macrophages expressing the GRIP1 protein at extremely low levels, and pharmacological disruption of the GR-GRIP1 interaction or transient introduction of GRIP1 restored RNA polymerase recruitment to target ISGs and the subsequent IFN response. Thus, type I IFN is a cytokine uniquely controlled by GR at the levels of not only production but also signaling through antagonism with the ISGF3 effector function, revealing a novel facet of the immunosuppressive properties of GCs.

Glucocorticoids (GCs) are a class of broadly immunosuppressive steroid molecules that are utilized as combative medicine for numerous inflammatory and autoimmune disorders, including asthma, rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), and many others. The therapeutic effects of GCs are largely attributed to their ability to suppress the production of important cytokines, including tumor necrosis factor alpha (TNF-α) (12, 18) and type I interferon (IFN) (19, 48, 52), which are proposed to be the primary mediators of RA and SLE pathogenesis, respectively. In the case of SLE, for example, the peripheral blood mononuclear cells (PBMCs) display a massive overexpression of conventional type I IFN target genes ("IFN signature"), which appears to correlate with disease activity and severity more than any other marker and is eradicated by administration of GCs (5–7, 29, 60).

GCs convey their actions by crossing the cell membrane and binding their cognate GC receptor (GR), a member of the nuclear receptor (NR) superfamily, which at steady state is maintained in a permissive conformation by molecular chaperones, such as hsp70 and hsp90 (47). Ligand binding facilitates the translocation of the cytosolic receptor to the nucleus, where liganded GR associates with specific DNA sequences known as GC response elements (GREs) and regulates transcription of target genes. In some cases, GR binds directly, usually as a homodimer, to specific palindromic DNA sequences ("simple" GREs). Conversely, for "tethering" GREs, GR does not itself bind DNA but is instead recruited by other DNA-bound transcription factors, such as nuclear factor κB (NF-κB) and activator protein 1 (AP1) (35). In contrast to simple GREs, which are commonly associated with transcriptional activation, GR occupancy of tethering GREs typically results in repression of target genes. The divergent ability of GR to activate or repress transcription depends upon many variables, including cell type, the DNA sequence to which GR is recruited, and the composition of available cofactors, which transduce signaling information from the activated GR to basal transcription machinery and chromatin. Of the latter, three members of the p160 family (SRC-1, GRIP1/TIF2/NCOA2/SRC-2, and RAC3/p/CIP/ACTR/AIBI/TRAM-1/SRC-3) are of critical importance in NR transcriptional regulation (59). Interestingly, while all three members of the p160 family are able to mediate transcriptional activation, GRIP1 alone has been implicated in corepression with GR at tethering GREs (17, 50, 51), with estrogen receptor (ER) alpha at a tethering TNF-α-RE (4), and with the myogenic regulatory factor MyoD (57).

Type I IFNs are produced by macrophages (MΦ) and other myeloid cells as an integral component of the host response to viral infection (27), and their production is suppressed by GCs (23, 45). Viral components bind Toll-like receptors (TLRs) to initiate a signaling cascade culminating in the activation of NF-κB and interferon regulatory factor 3 (IRF3), which then work in concert to induce the transcription and subsequent secretion of type I IFNs, specifically beta IFN (IFN-β) and alpha 4 IFN (IFN-α4) (38). These IFN subsets initiate an amplification loop by

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binding the IFN-α/β receptor, which induces activation of the constitutively associated tyrosine kinases Tyk2 and Jak1 and the subsequent recruitment and phosphorylation of STAT1 and STAT2 (37). A third transcription factor, IRF9, associates with the STAT1/2 heterodimer through interactions with STAT2, and the resultant trimeric complex, ISGF3, then binds to its cognate IFN-stimulated response elements (ISREs) on the DNA and activates transcription of the type I IFN-stimulated genes (ISGs). Treatment of MΦ with a synthetic GC, dexamethasone (Dex), antagonizes the activity of the NF-κB and IRF3 complexes induced by TLRs (46, 49); thus, it is possible that GC-mediated inhibition of ISG expression and eradication of the IFN signature are in part a consequence of the suppression of type I IFN gene transcription. Interestingly, while a wealth of evidence points to GC-mediated inhibition of cytokine production, much less is known about the effects of GR on the signal transduction pathways initiated by cytokines at the cell surface. It has been shown that GR synergizes with prolactin-activated STAT5 and with interleukin 6 (IL-6)-activated STAT3 (34, 36, 54); however, the mechanisms of synergy are unclear. Unexpectedly, we found that ISG expression in murine MΦ induced by exogenously provided type I IFN was strongly attenuated by cotreatment with Dex, suggesting that the IFN signaling pathway itself is under GC control. Here, we assessed the effects of GCs on type I IFN-Jak/STAT signaling and dissected the transcriptional regulatory mechanism responsible for the GC sensitivity of the IFN-dependent gene expression. Our findings reveal previously unexplored functional interactions between the GR and IFN pathways, which may underlie the immunosuppressive properties of GCs.

MATERIALS AND METHODS

Plasmids. IP10 promoter-luciferase constructs (−533-Luc, −533-Luc.mt [ISREmt], and p31x2-Luc [2xISRE]), β-actin-LacZ, pET-GRIP1 3-RD, pCDNA3-GRIP1, and pCDNA3-GRIP1 N1007 were previously described (49–51). pGEX.IRF9 was generated by excising IRF9 from pCDNA3 with BamHI/NotI and subcloning it into the BamHI/NotI sites of pGEX4T-1 (Amersham-Pharmacia). pGEX.IRF9.N145 was generated from pGEX.IRF9 by incorporating an internal NotI site by site-directed mutagenesis (QuikChange, Stratagene) using mutagenic primers F (5′-CAGCACAGTTC TGCGGCCGCTGAGAGGAAGGAGG-3′) and R (5′-CCTCCTTCCTCTC AGCGGCCGCAGAACTGTGCTG-3′). The C-terminal IRF9 fragment was excised by digestion with NotI, and the plasmid was religated.

pGEX.IRF9.127C was generated from pGEX.IRF9 by incorporating an internal BamHI site by site-directed mutagenesis using mutagenic primers F (5′-CGTCTCTG GCCAGCCAGGGATCCAGAAAGTACCATCAAAGC-3′) and R (5′-GCTTTGA TGGTACTTTCTGGATCCCTGGCTGGCCAGAGACG-3′). The N-terminal IRF9 fragment was excised by digestion with BamHI, and the plasmid was religated.

pGEX.IRF9.213C was generated from pGEX.IRF9 by incorporating an internal XbaI site by site-directed mutagenesis using mutagenic primers F (5′-CTGGAGTTTC TGCTTCCTCTAGAGCCAGACTACTCACTG-3′) and R (5′-CAGTGAGTAGTC TGGCTCTAGAGGAAGCAGAAACTCCAG-3′). The N-terminal IRF9 fragment was excised by digestion with XbaI, and the plasmid was religated.

pGEX.IRF9.127-208 was generated from pGEX.IRF9.127C by incorporating an internal XhoI site by site-directed mutagenesis using mutagenic primers F (5′-GGAATTCCCGGGTCGACTGAGTTTCTGCTTCC-3′) and R (5′-GGAA GCAGAAACTCAGTCGACCCGGGAATTCC-3′). The IRF9 fragment was excised by digestion with BamHI/XhoI and subcloned into the BamHI/XhoI sites of pGEX4T-1.

To generate −533-Luc-κB1, −533-Luc-κB2, and −533-Luc-AP1, the κB1, κB2, or AP1 sites were disrupted by site-directed mutagenesis with the primers κB1-F (5′-GC CCTCGGTTTACGGGAAGCTTCCCTCGGGTTGCG-3′) and κB1-R (5′-CGCAA CCCGAGGGAAGCTTCCCGTAAACCGAGGGC-3′), κB2-F (5′-GGAGCACAA GAGGGGAGAGCCGAATTCCAAGTTCATGGG-3′) and κB2-R (5′-CCCATGA ACTTGGAATTCGGCTCTCCCCTCTTGTGCTCC-3′), and AP1-F (5′-GGTTGC GGAGCCTTGCGCAGTCACCTCCAAAGTC-3′) and AP1-R (5′-GACTTTGGAG GTGACTGCGCAAGGCTCCGCAACC-3′), respectively.

Cell culture and transfections. CV-1 green monkey kidney fibroblasts and murine RAW 264.7 MΦ-like cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS). Mouse 3T3 fibroblasts were cultured in DMEM-10% FBS supplemented with MEM nonessential amino acids and 1.75 μM 2-mercaptoethanol. Bone marrow-derived MΦ (BMMΦ) were prepared from 8-week-old C57BL/6 mice as described in reference 49, except L929 cell-conditioned medium (LCCM) was used for the 6-day MΦ expansion; cells were then scraped and reseeded into DMEM-20% FBS for 24 h prior to treatment.

For small interfering RNA (siRNA), 1.8×10^7 RAW 264.7 cells were transfected with 200 μmol control or GRIP1 siRNA (Qiagen) using the Cell Line Nucleofector kit V (Amaxa catalog no. VCA-1003) as per the manufacturer's instructions. Cells were allowed to recover for 18 h before treatment.

RAW 264.7 cells were transfected in 6-well plates (5×10^5 cells/well) using the GenePorter 3000 system (Genlantis) with 275 μl GP3K diluent and 28 μl GP3K reagent per well in FBS-free DMEM, as per the manufacturer's instructions, and refed with DMEM-10% FBS 5 h posttransfection. Cells were allowed to recover for 24 h before treatment.

CV-1 cells were transfected in 24-well plates in FBS-free media using 1 μl Lipofectamine and 2 μl Plus (Invitrogen) per well and refed with DMEM-10% FBS 3 h later. The following day, cells were treated (see the figure legends) and harvested for luciferase and β-galactosidase assays 6 h later (51).

Protein purification and *in vitro* binding assays. Glutathione *S*-transferase (GST)- and His-tagged proteins were generated in *Escherichia coli* as described previously (49), except the expression was induced with 0.2 mM IPTG (isopropyl-β-D-thiogalactopyranoside) for 5 h at 25°C. GRIP1 derivatives and the GR were produced using the coupled *in vitro* transcription/translation system (Promega) in the presence of [^{35}S]methionine and (for the GR) 1 μM Dex, and binding assays were performed in the presence of 0.05% NP-40, as described previously (49).

Western blotting. For immunoprecipitations, 3T3 fibroblasts or RAW 264.7 cells were cultured in 150-mm dishes and treated as described in the figure legends, and protein extracts were prepared as described in reference 49. Twenty percent of each clarified extract was boiled in 2× SDS sample buffer to generate whole-cell extracts (WCE), while the rest was incubated with 4 μg of anti-GRIP1 C-terminal antibody (Santa Cruz catalog no. sc-6976) at 4°C overnight, after which 100 μl of a 50% slurry of protein A/G Plus agarose was added and incubation continued for 1 h at 4°C. Immunoprecipitates were collected, and immunoblotting was performed as described in reference 49. Blotting antibodies used were STAT1 (Santa Cruz catalog no. sc-346), STAT2 (Santa Cruz catalog no. sc-22816), pY701-STAT1 (Cell Signaling catalog no. 9171), pY689-STAT2 (Millipore catalog no. 07-224), pS727-STAT1 (Cell Signaling catalog no. 9177), STAT3 (Santa Cruz catalog no. sc-482), ERK (Santa Cruz catalog no. sc-94), TIF2 (BD Transduction Laboratories catalog no. 610985), GRIP1 (Abcam catalog no. 10491), IRF9 (Santa Cruz catalog no. sc-10793), and GST (Thermo Scientific catalog no. 3001). Primary antibodies were detected using horseradish peroxidase (HRP)-conjugated anti-mouse or anti-rabbit IgG (Promega).

Chromatin immunoprecipitations. Chromatin immunoprecipitation (ChIP) assays with BMMΦ, RAW 264.7 cells, and 3T3 mouse fibroblasts were performed as described previously (2). Antibodies used were STAT1, STAT2, Pol2, GRIP1 (Santa Cruz catalog no. sc-346, sc-22816, sc-899, and sc-6976, respectively), AcH3 (Millipore catalog no. 06-599), GR N499 (51), or normal IgG (Santa Cruz catalog no. sc-2027). Primer pairs for target genes are listed at http://www.hss.edu/files/Supplementary_Figures_and_table_1.pdf.

RNA isolation and real-time PCR. Total RNA was isolated using the RNeasy minikit (Qiagen). Random primed cDNA synthesis and quantitative PCR (qPCR) were performed as described previously (49), equalizing total RNA input. GAPDH (glyceraldehyde-3-phosphate dehydrogenase) or β-actin was used as a normalization control. Primer pairs are listed at http://www.hss.edu/files/Supplementary_Figures_and_table_1.pdf.

Adenovirus-mediated GRIP1 knockdown. BMMΦ from mice bearing a floxed GRIP1 allele (GRIP1$^{flox/flox}$) were cultured as described above. On day 6, 1.5×10^6 cells were infected at a multiplicity of infection (MOI) of 1:1,000 with Ad5-cytomegalovirus (CMV)-green fluorescent protein (GFP) (Ad-GFP) or Ad5-CMV-Cre (Ad-Cre) (Vector Development Labs) in DMEM plus 0.5% FBS for 14 h. Cells were refed with DMEM plus 20% FBS and allowed to recover for 30 h prior to treatment.

RESULTS

Type I IFN target gene expression is directly inhibited by Dex. Unlike cytokine gene expression, cytokine signaling is reportedly unaffected and in some cases even potentiated by

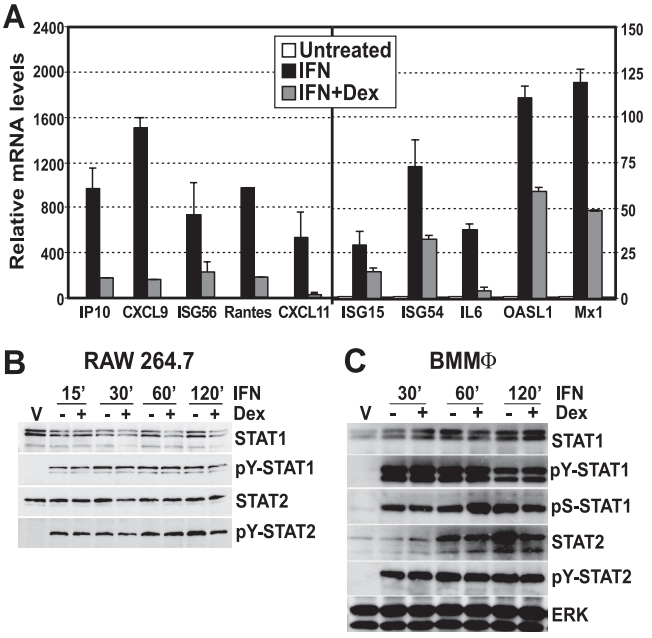

FIG. 1. Dex inhibits IFN-induced gene expression downstream of Jak/STAT pathway activation. (A) Inhibition of IFN-induced gene expression by Dex. BMMΦ were treated for 1 h (ISG56, ISG15, ISG54, OASL1, Mx1), 2 h (IP10, CXCL9, CXCL11, IL-6), or 4 h (Rantes) with vehicle (untreated) or 500 U/ml IFN with or without 100 nM Dex, as shown. mRNA abundance of ISGF3 target genes was determined by qPCR, with GAPDH as the normalization control, and expressed relative to untreated cells (control = 1). Error bars represent ±standard errors of the mean (SEM). Results are from at least eight independent experiments. (B and C) Type I IFN-induced phosphorylation of STAT1 and STAT2 is Dex resistant. RAW 264.7 cells or BMMΦ were cultured for the indicated times in the presence of 500 U/ml IFN with (+) or without (−) 100 nM Dex. STAT1 and STAT2 expression and activation by tyrosine (Y701 and Y690, respectively) or serine (S727, STAT1 only) phosphorylation was assessed by immunoblotting.

GR (36, 54, 55). Treatment of murine bone marrow-derived MΦ (BMMΦ) with type I IFN induced, as expected, the expression of a series of established ISGs (encoding IP10, CXCL9, ISG56, Rantes, CXCL11, ISG15, ISG54, IL-6, OASL1, and Mx1). To our surprise, this induction was markedly attenuated by concurrent administration of Dex (Fig. 1A). As expected, the level of induction of different genes depended on the IFN dose and duration of treatment (see Fig. S1A and B at http://www.hss.edu/files/Supplementary_Figures_and_table_1.pdf). Similarly, downregulation by Dex varied in magnitude and duration but was nonetheless observed for each ISG analyzed. This suppression of gene induction was specific to type I IFN targets, as gamma IFN (IFN-γ)-dependent induction of IRF1 was Dex resistant (see Fig. S1C at http://www.hss.edu/files/Supplementary_Figures_and_table_1.pdf).

A similar dramatic inhibition by Dex was observed when nascent unprocessed ISG transcripts were analyzed using intronic primers (see Fig. S2A at http://www.hss.edu/files/Supplementary_Figures_and_table_1.pdf), ruling out the effects on downstream steps such as mRNA processing or stability. In some hematopoietic cells, GCs indirectly obstruct the activation of STAT proteins through the induction of intermediary genes, such as the suppressor of cytokine signaling (SOCS1), which interferes with the function of STATs (14). Thus, the sensitivity of ISGs to Dex treatment could result from the induction of a putative IFN-signaling inhibitor by the activated GR. Additionally, in T cells, GCs were proposed to alter the expression of upstream signaling components, ultimately affecting STAT4/5 activation (10, 24). However, concurrent treatment with cycloheximide under conditions previously shown to block *de novo* protein synthesis in macrophages (30, 40) did not relieve Dex-dependent inhibition (see Fig. S2B at http://www.hss.edu/files/Supplementary_Figures_and_table_1.pdf), indicating a direct effect of GR on the preexisting components of the IFN signaling pathway.

Jak/STAT pathway activation is unaffected by Dex treatment. In principle, GR can modulate a given signal transduction pathway by directly altering the activities of kinases or phosphatases. To determine whether GR affects Jak/STAT pathway activation, we assessed STAT1/2 tyrosine phosphorylation (Y701 and Y690, respectively) in response to IFN versus IFN plus Dex. STAT proteins were rapidly phosphorylated in response to IFN in both RAW 264.7 cells and primary BMMΦ (Fig. 1B and C), and this activation mark was unaffected by Dex for up to 2 h of treatment. Similarly, IFN-dependent phosphorylation of STAT1 S727, proposed to be important for full STAT1 activation (21), was also Dex resistant (Fig. 1C). Thus, activation of the Jak/STAT pathway by type I IFN appears to be refractory to GC treatment. In addition, as ISGF3 nuclear localization is controlled by IFN-dependent STAT1/2 phosphorylation (33), we assayed the subcellular distribution of STAT1/2 in BMMΦs. Consistent with the results of immunoblotting, indirect immunofluorescence revealed a similar pattern in cells treated with IFN and those treated with IFN plus Dex (data not shown).

IFN induction and Dex inhibition of the ISGF3 function is mediated by ISREs. Many ISGs contain binding elements for and are regulated by multiple transcription factors. Because STAT1/2 activation was unaffected by Dex, we questioned whether stimulation and inhibition of ISG expression are specifically mediated by the ISGF3 binding sites, ISREs. We generated a series of IP10-derived luciferase (Luc) reporter constructs and tested their responses to IFN and Dex in a cell-based reporter assay in IFN-responsive CV-1 cells (Fig. 2A). As expected, IFN treatment induced the wild-type (WT) reporter activity, while mutation of the ISRE abrogated IFN-mediated induction (Fig. 2B). Although the basal activity of this ISREmt reporter was also significantly reduced relative to that of the WT, phorbol myristate acetate (PMA) ester, a strong activator of AP1 and NF-κB, considerably induced Luc activity (data not shown), suggesting that the lack of IFN responsiveness was not due to the global disruption of the reporter. In contrast, constructs with an intact ISRE but mutated AP1 and NF-κB elements, either individually (Fig. 2C) or in combination (Fig. 2B, 3° mt), were induced by IFN and inhibited by Dex, similar to the WT. Furthermore, a simplified reporter containing only a dimerized, IFN-β-derived ISRE controlling Luc expression was strongly activated by IFN, and Dex cotreatment abrogated the response (Fig. 2B). Thus, the ISGF3-binding ISRE was necessary and sufficient for the IFN induction and GC inhibition of ISG-derived reporters.

Dex inhibits IFN-induced ISGF3 transcription complex assembly at ISG promoters in MΦ. The results of the cell-based

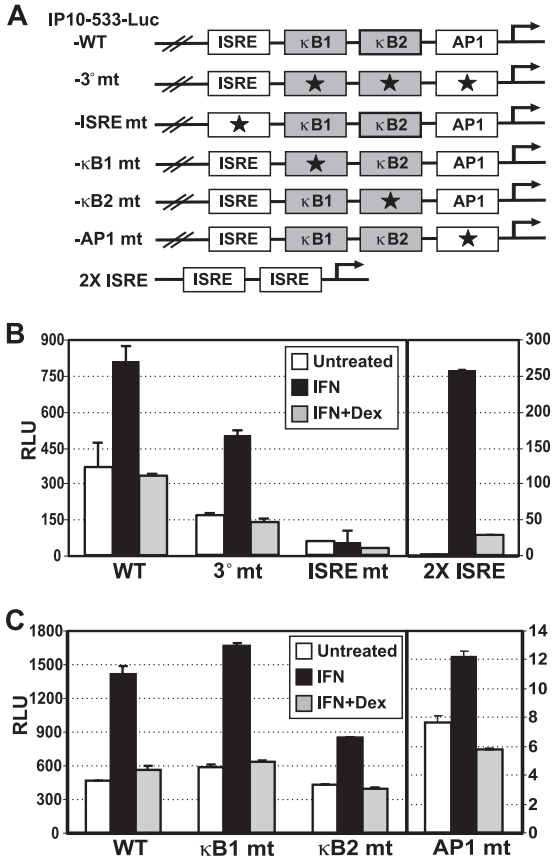

FIG. 2. IFN induction and Dex inhibition are mediated by ISREs. (A) Diagram of a series of IP10-derived luciferase reporters, with WT or mutated (stars) promoter elements and a dimerized, IFN-β-derived ISRE reporter (2xISRE). (B and C) CV-1 cells (10^5) were transfected with 200 ng pCDNA3.rGR, 35 ng pCMV-LacZ, and 200 ng of the indicated reporter constructs listed in panel A and treated the following day for 6 h as indicated. Luciferase activity was normalized to β-galactosidase activity (as a measure of transfection efficiency) and expressed as relative luminescence units (RLU). Error bars represent ±SEM. Results are from five independent experiments.

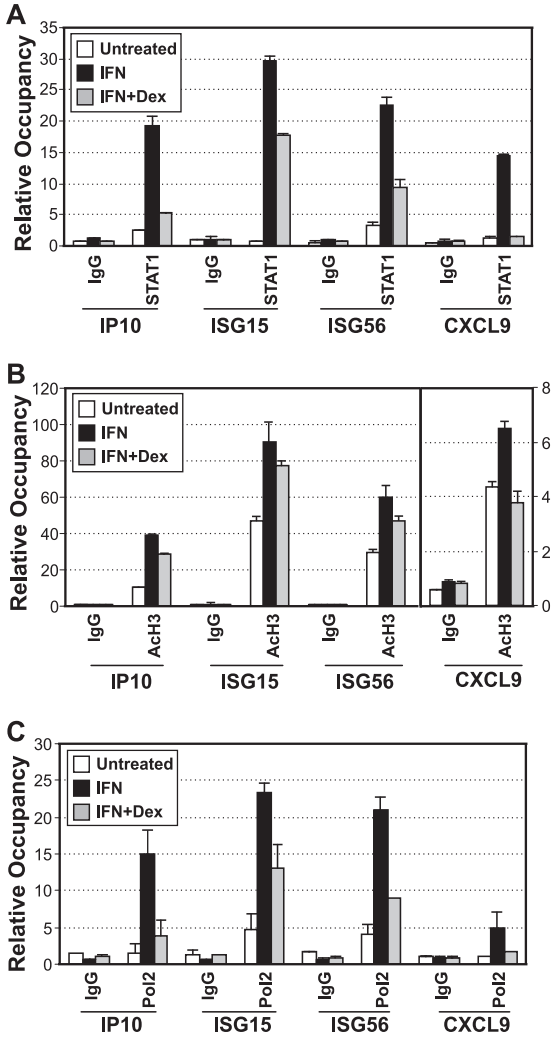

FIG. 3. Dex inhibits IFN-induced transcription complex assembly. BMMΦ were treated as indicated for 30 min. ChIP assays were performed using antibodies to STAT1 (A), H3AcK9/K14 (B), Pol2 (C), or the isotype-matched control IgG (A to C). Occupancy was determined by qPCR amplification over ISRE (A) or TSS (B and C) regions of the indicated target genes, normalized to the internal control (45S), and expressed relative to the mean signal obtained from cells precipitated with control IgG (set to 1). Error bars represent ±SEM. Results are from at least three independent experiments.

reporter assays suggested that ligand-activated GR could be targeting transcription complex assembly or function at ISG promoter elements. We therefore examined by chromatin immunoprecipitation (ChIP) whether Dex treatment affects the occupancy of the ISGF3 complex at ISREs of IFN-regulated genes in primary BMMΦ. In response to IFN, STAT1 occupancy of ISREs of model ISGs, including IP10, ISG15, ISG56, and CXCL9, increased dramatically, and cotreatment with Dex attenuated this increase for all genes tested (Fig. 3A). A similar pattern was observed for STAT2 (see Fig. S3A at http://www.hss.edu/files/Supplementary_Figures_and_table_1.pdf), although overall ChIP signals for STAT2 were modest (relative to those of the IgG control), likely due to the quality of the STAT2 antisera available. It is also possible that the IFN-inducible recruitment of STAT1 and STAT2 to ISG promoters is nonstoichiometric.

Next, we assessed whether the changes in ISGF3 occupancy at ISREs in BMMΦ correlated with alterations in markers of transcriptional activation, such as acetylation of lysines 9 and 14 of histone H3 at ISG regulatory regions. As shown in Fig. 3B, the basal levels of H3AcK9/14 in BMMΦ were highly variable between the individual ISGs at transcription start sites (TSS). Indeed, basal H3 acetylation at the IP10 promoter was only moderately above the background of normal IgGs, whereas H3 at the ISG15 and ISG56 promoters was strongly acetylated (45- and 30-fold over the background, respectively) in untreated cells. Nonetheless, in all cases, some additional acetylation occurred in conjunction with IFN treatment and was diminished by Dex cotreatment (Fig. 3B). No change in the total level of histone H3 was observed in response to either IFN or IFN plus Dex in any of the genes tested (not shown).

The definitive mark of transcription initiation is the assembly of basal transcriptional machinery, including RNA polymerase II (Pol2), into the preinitiation complex at TSS near target promoters. Our ChIP assays revealed that Pol2 occu-

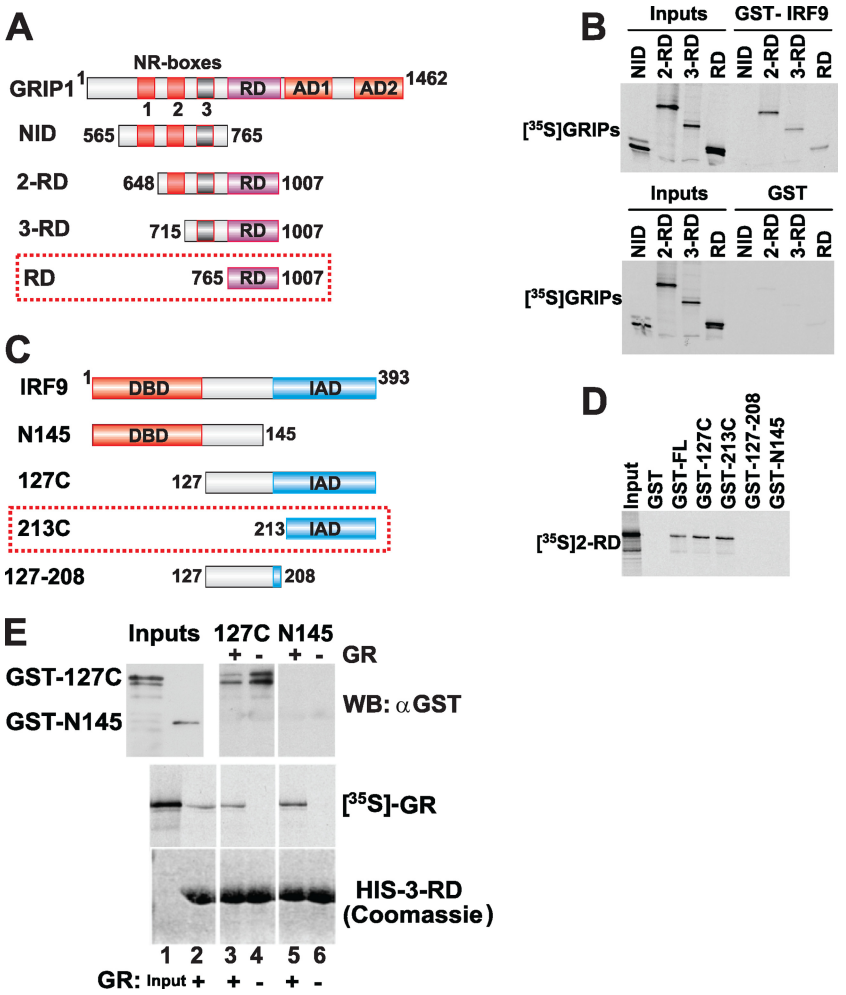

FIG. 4. GRIP1 and IRF9 interact *in vitro* in a GR-sensitive manner. (A and C) Domain diagrams of full-length GRIP1 (A) and IRF9 (C) and their derivatives produced *in vitro* and recombinantly in *E. coli* as GST fusion proteins, respectively. (B and D) Mapping the interacting surface on GRIP1 and IRF9. (B) ^{35}S-radiolabeled GRIP1 derivatives listed in panel A were tested for their ability to interact with full-length recombinant GST-IRF9 (top) or GST alone (bottom). (D) Binding assays were performed between ^{35}S-GRIP1 2-RD and GST-IRF9 derivatives listed in panel C. (E) The GRIP1-IRF9 interaction is disrupted by GR. His-tagged GRIP1 3-RD immobilized on affinity resin was incubated with GST-IRF9 127C (lanes 3 and 4) or N145 (lanes 5 and 6), in the presence or absence of ^{35}S-GR, as indicated. Dex (1 μM) was present in all reactions. GR binding to 3-RD was verified by autoradiography (middle), and IRF9 binding was assessed by immunoblotting with GST-specific antibodies (top). Immobilized 3-RD was visualized by Coomassie blue staining (bottom).

pancy was robustly induced by IFN at the ISG TSS regions. This increase was largely blocked by Dex (Fig. 3C), indicating that GR activation attenuates transcription initiation of these genes.

GR is known to interfere with transcriptional activation by tethering to other DNA-bound regulators and sterically blocking their transactivation domains or preventing the recruitment or activation of the preinitiation complex (22, 35). However, consistent with previous observations (3), we failed to detect a physical interaction *in vitro* between GR and any components of the ISGF3 (not shown), indicating that a tethering mechanism of inhibition is unlikely to operate at the ISREs. Furthermore, no apparent GR occupancy was observed at ISG promoters in RAW 264.7 cells treated with IFN or IFN plus Dex; as expected, GR was recruited to its established target gene, GILZ, in a Dex-dependent manner (see Fig. S3B at http://www.hss.edu/files/Supplementary_Figures_and_table_1.pdf).

IRF9 and GRIP1 interact *in vitro*. Because ISGF3 nuclear localization was similar in cells treated with IFN and cells treated with IFN plus Dex, we reasoned that a reduction in apparent ISGF3 occupancy at ISREs may reflect a shorter residence time of the complex on the DNA, perhaps due to Dex-dependent destabilization or loss of associated cofactors. Indeed, activated GR has been proposed to displace essential coregulators from other transcription factors (31). Studies in our lab have shown that GR antagonized IRF3 activity induced by the TLR3 agonists through depleting the p160 family member GRIP1, which is required for IRF3-dependent ISG transcription (49). Because the IRF association domain (IAD) of IRF3, responsible for binding GRIP1, is 21% identical (35% similar) to that of IRF9, we investigated the possibility of a protein-protein interaction between IRF9 and GRIP1. We produced *in vitro* a series of GRIP1 derivatives centered across its IRF3-binding repression domain (RD), including the NR-

interacting domain (NID; amino acids [aa] 565 to 765), 2-RD (aa 648 to 1007, containing NR boxes 2 and 3 and the RD), 3-RD (aa 715 to 1007, containing NR box 3 and the RD), and the RD alone (aa 765 to 1007) (Fig. 4A), and tested them for their ability to bind purified recombinant full-length GST-IRF9. We found that all but the NID interacted with GST-IRF9 but not with the GST control; as in our earlier studies with IRF3 or Suv4-20h1 (16, 49), RD bound IRF9 less well than 3-RD (Fig. 4B). Given secondary structure predictions for isolated RD and the fact that the N-terminal 50 aa upstream of RD (which differentiate it from 3-RD) do not enable NID-IRF9 interaction, our results suggest that RD is the major surface of GRIP1 interacting with IRF9 and that the N-terminal 50-aa extension serves to stabilize the RD conformation.

To identify the IRF9 domain(s) responsible for GRIP1 binding, we generated a series of GST-IRF9 deletion mutants and tested their ability to bind GRIP1 2-RD *in vitro*. These included N145 (aa 1 to 145, containing the N-terminal DNA binding domain [DBD] and a portion of the linker region), 127C (aa 127 to 393, containing the linker region and the C-terminal IAD), 213C (aa 213 to 393, containing the IAD only), or 127-208 (containing the linker region with a small segment of the IAD) (Fig. 4C). N145 did not bind 2-RD (Fig. 4D), suggesting that, similar to IRF3, the IRF9 DBD is not sufficient for the GRIP1-IRF9 interaction. Conversely, 127C and 213C, but not 127-208, were both able to bind 2-RD. Hence, the GRIP1-interacting region encompasses the IRF9 IAD (aa 213 to 393) and excludes the N-terminal DNA binding and linker domains.

GR binds GRIP1 via NR box 3 (20) immediately adjacent to the IRF9-binding RD, suggesting that GR binding may sterically hinder the formation of the GRIP1-IRF9 complex. To assess whether the GRIP1-IRF9 interaction was affected by GR, we utilized 3-RD, the minimal GRIP1 construct able to bind both IRF9 and GR. As expected, in the presence of 1 μM Dex, *in vitro* transcribed/translated GR-bound recombinant His-tagged GRIP1 3-RD immobilized on metal affinity resin (Fig. 4E, lanes 1 and 2). Consistent with data shown in Fig. 4D, IRF9 127C but not IRF9 N145 bound GRIP1 (Fig. 4E, top, lanes 4 and 6); furthermore, the GRIP1 3-RD–IRF9 127C interaction was potently inhibited in the presence of GR (Fig. 4E, top, lanes 3 and 4). Thus, agonist-activated GR directly disrupts the GRIP1-IRF9 complex.

GRIP1 functions as an ISGF3 coactivator in MΦ. Transcription initiation is a stepwise process involving the sequential recruitment of multiple coregulators which perform diverse functions, including covalent modifications of histones and chromatin, recruitment of basal machinery and Pol2, as well as stabilization of the DNA-bound regulator complex itself by facilitating intermolecular interactions and/or preventing its proteosomal degradation. Given the physical interaction between GRIP1 and IRF9, we speculated that GRIP1 may serve as an ISGF3 coactivator, in which case disruption of the GRIP1-IRF9 interaction could alter ISGF3 transcriptional activity by disabling any of the above mechanisms. To test this hypothesis, we used the GRIP1.N1007 derivative (50), which retains the IRF9-binding RD but lacks the AD1/2 responsible for recruiting the secondary coactivators CBP/p300 and CARM1 (42). When cotransfected into CV-1 cells along with the minimal ISRE-driven Luc reporter, GRIP1.N1007 inhibited IFN-induced reporter activity in a dose-dependent manner (Fig. 5A), presumably by binding to IRF9 and displacing endogenous full-length GRIP1 from the ISGF3 complex. Because a dominant negative approach may suffer from nonspecific effects of overexpression, we investigated whether knockdown of endogenous GRIP1 with siRNA would affect ISG expression in RAW 264.7 MΦ-like cells. We found that relative to cells transfected with scrambled siRNA (siC), depletion of GRIP1 protein (siG) potently attenuated IFN induction of all ISGs tested (Fig. 5B).

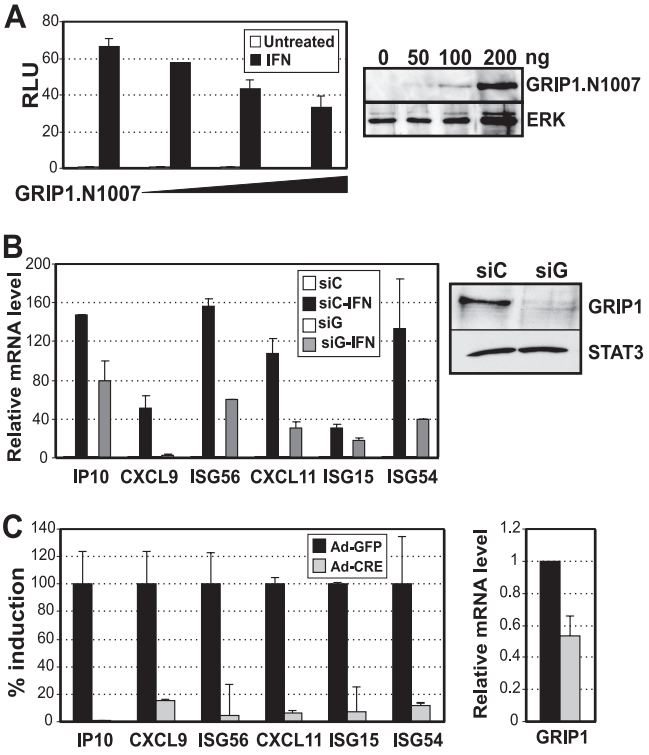

FIG. 5. IFN-induced gene expression is dependent upon the presence of active GRIP1. (A) GRIP1.N1007 overexpression attenuates IFN-induced transcription. CV-1 cells (10^5) were transiently transfected with 35 ng pCMV-LacZ, 200 ng 2xISRE-Luc, and increasing amounts (0, 50, 100, and 200 ng) of pCDNA GRIP1.N1007 or with pCDNA3 to equalize the total amount of transfected DNA. The following day, cells were treated for 6 h with 500 U/ml IFN, and whole-cell lysates were assayed for luciferase activity (exactly as described for Fig. 2) (left) or GRIP1 expression by immunoblotting (right). (B) siRNA depletion of GRIP1 antagonizes IFN-dependent ISG induction. RAW 264.7 cells (2×10^6) were transfected with 3 μg of siRNA against GRIP1 (siG) or scrambled RNA (siC) as a negative control. Eighteen hours later, cells were treated with 500 U/ml IFN for 6 h. The GRIP1 protein level was analyzed by immunoblotting with anti-STAT3 blot to verify equal loading (right), and mRNA expression levels of target genes were analyzed by qPCR (left), as described for Fig. 1. (C) Adenovirus-mediated GRIP1 knockdown in primary MΦ attenuates the IFN response. Primary BMMΦ were derived from $GRIP1^{flox/flox}$ mice as described in Materials and Methods and infected with adenovirus-expressing Cre recombinase (Ad-Cre) or the control GFP (Ad-GFP). mRNA levels of the indicated genes were analyzed by qPCR, as described for Fig. 1. ISGs are expressed as a percentage of IFN induction in Ad-GFP-infected cells (100%). GRIP1 is expressed relative to the mean signal obtained from cells infected with the control Ad-GFP (set to 1). Error bars represent ±SEM. Results are from at least four independent experiments.

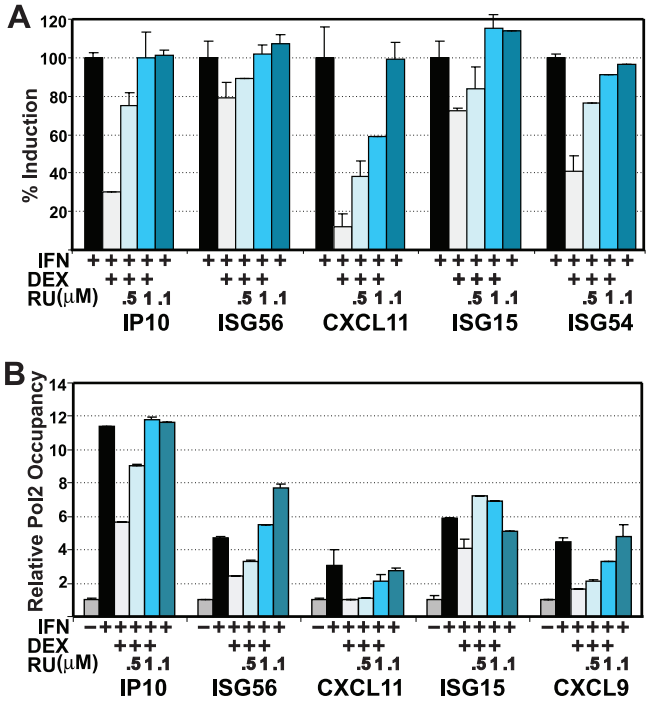

FIG. 6. RU486 relieves Dex-mediated inhibition of ISG transcription. (A) BMMΦ were treated for 2 h with 500 U/ml IFN with or without 100 nM Dex, with or without the indicated concentrations of RU486 (RU). mRNA abundance of ISGF3 target genes was determined by qPCR, with β-actin as the normalization control, and is expressed as a percentage of induction by IFN alone (100%). (B) BMMΦ were treated as indicated for 30 min. ChIP assays were performed using Pol2 antibodies or isotype-matched control normal IgG (not shown). Occupancy was determined by qPCR amplification over TSS regions of the indicated target genes, as described for Fig. 3. Error bars represent ±SEM.

To determine the role of GRIP1 in primary cells, we utilized an *ex vivo* knockdown approach in BMMΦ derived from mice bearing a floxed GRIP1 allele (graciously provided by Pierre Chambon [25]). GRIP1$^{flox/flox}$ BMMΦ were infected in culture with an adenovirus expressing either Cre recombinase (Ad-Cre) or the control GFP (Ad-GFP). Indeed, Ad-Cre infection significantly attenuated GRIP1 expression compared to that in Ad-GFP-infected BMMΦ (Fig. 5C, right). Strikingly, the induction of a panel of type I IFN target genes was nearly abrogated in GRIP1-depleted MΦ (Fig. 5C, left). Furthermore, ISG expression in primary BMMΦ was considerably more sensitive to the loss of GRIP1 than in RAW 264.7 cells, likely due to the very low levels of GRIP1 protein present in these cells. Combined, these results establish a critical role for endogenous GRIP1 in MΦ in the transcriptional activation of ISGs via the IFN-Jak/STAT pathway.

Pharmacological antagonism of GR-GRIP1 binding restores IFN-dependent gene expression in MΦ. The p160 family members, including GRIP1, interact with GR in conjunction with ligand binding. Specifically, GR agonists, such as Dex, induce a conformational change in the receptor ligand binding domain (LBD), promoting the formation of the activation function 2 (AF2) surface, which then recruits GRIP1. In contrast to full agonists, the partial antagonist RU486 precludes the formation of AF2 and, thus, p160 recruitment (11). If inhibition of ISGF3 activity by Dex occurs due to the sequestration of GRIP1 from IRF9 by the agonist-bound GR, then competitive antagonism by excess RU486 will displace Dex from the GR LBD, allowing for GRIP1 release and interaction with IRF9, thereby restoring ISG expression. Figure 6A demonstrates that, on its own, RU486 treatment of BMMΦ did not affect the IFN induction of any ISG tested and lifted Dex-imposed inhibition in a dose-dependent manner. Furthermore, we observed a remarkable correlation between the mRNA expression data and the effects of GR ligands on preinitiation complex assembly at ISGs. As assessed by ChIP, IFN-induced Pol2 occupancy of ISG TSS was largely unaffected by RU486, while the Dex-dependent reduction in occupancy was reversed in a dose-dependent manner (Fig. 6B). Taken together, these data suggest a scenario in which Dex-mediated inhibition of IFN-induced gene expression is caused by sequestration of GRIP1 by activated GR from its duties as an ISGF3 coactivator.

Glucocorticoid regulation of IFN signaling is cell type specific. To examine whether GC-dependent regulation of IFN signaling is a common feature of different cell types, we assessed the IFN response in murine 3T3 fibroblasts, which express all components of the GR and IFN pathways endogenously. IFN treatment resulted in a potent time-dependent induction of a panel of ISGs at 0.5, 1, and 2 h (Fig. 7A and data not shown); surprisingly, however, the induction appeared to be completely Dex resistant. The lack of Dex response did not reflect a disruption of the GC pathway, as GR is well expressed in mouse fibroblasts (Fig. 7B, middle) and, as expected, is able to activate the GC-responsive genes GILZ and IGFBP1 (Fig. 7A, right).

Interestingly, the level of GRIP1 protein was strikingly different between the murine cell types examined, with fibroblasts expressing a significantly greater amount of GRIP1 relative to BMMΦ or even RAW 264.7 cells (Fig. 7B, top). We speculated that perhaps the higher GRIP1 expression in 3T3 cells allows for its utilization by both GR and ISGF3, thereby relieving the inhibitory effect of GR on ISG expression. Furthermore, this elevated expression should enable us to employ ChIP of IFN target genes to visualize GRIP1, which in MΦ was below the level of detection. Indeed, a C-terminal antibody to GRIP1 revealed an IFN-dependent increase in GRIP1 occupancy at ISREs of several target ISGs, which was largely unaffected by Dex (Fig. 7C, left). Similarly (and in stark contrast to our observations in MΦ [Fig. 3B]), a robust IFN-induced Pol2 recruitment to the TSS of these genes was also refractory to Dex treatment (Fig. 7C, right). Overall, we did not expect a dramatic increase in the apparent GRIP1 occupancy in response to IFN in 3T3 cells, as GRIP1 is constitutively nuclear and IRF9 is largely nuclear even in the absence of IFN treatment; consistently, endogenous GRIP1 coimmunoprecipitated IRF9 from mouse fibroblasts prior to IFN stimulation, and this complex was Dex resistant (see Fig. S4 at http://www.hss.edu/files/Supplementary_Figures_and_table_1.pdf). It should be noted that the GRIP1 C-terminal antibody used for coimmunoprecipitation (coIP) and ChIP is far less effective for GRIP1 IP than other commercial antibodies raised to GRIP1 epitopes overlapping the IRF9-interacting RD (data not shown); importantly, however, this was the only antibody capable of co-

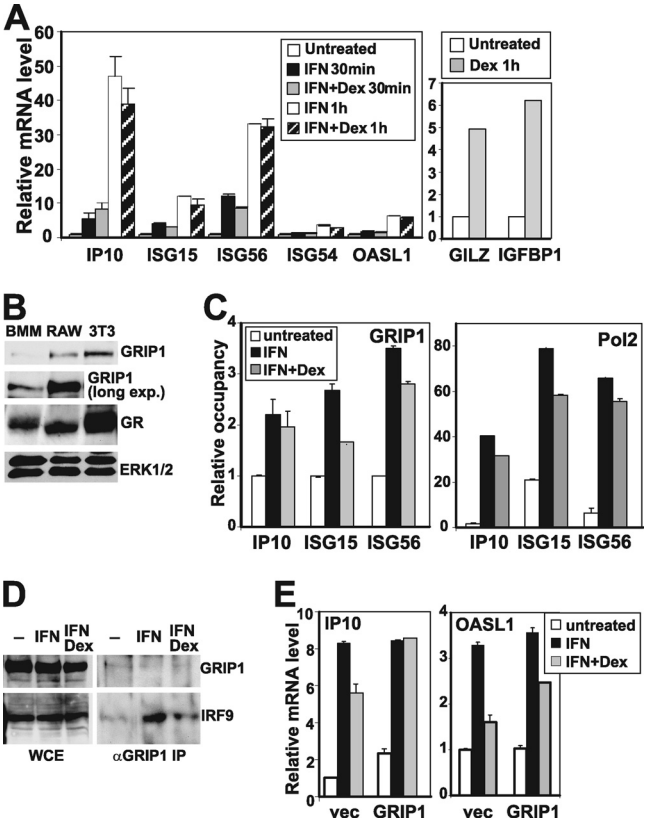

FIG. 7. The effect of glucocorticoids on ISG expression depends on the GRIP1 protein level in a cell. (A) ISG expression in 3T3 mouse fibroblasts is Dex resistant. 3T3 cells were treated for the indicated times with 500 U/ml IFN with or without 100 nM Dex (left) or Dex alone (right), and the mRNA abundance of the indicated genes was determined by qPCR, with β-actin as the normalization control. (B) GRIP1 protein level varies dramatically between cell types. An equivalent amount of whole-cell extracts (WCE) from BMMΦ, RAW 264.7 cells, or 3T3 fibroblasts were fractionated by SDS-PAGE, and the expression of GRIP1, GR, and ERK1/2 (as a loading control) was assessed by immunoblotting. (C) IFN-dependent GRIP1 and Pol2 recruitment to ISGs in fibroblasts is Dex resistant. 3T3 cells were treated for 1 h as indicated, and GRIP1 and Pol2 occupancy at the ISRE or TSS regions, respectively, of indicated genes was determined by qPCR, normalized to the internal control (45S), and expressed relative to the mean signal obtained from cells precipitated with the control IgG (set to 1). (D) GRIP1-IRF9 interaction in RAW 264.7 cells is sensitive to Dex. RAW 264.7 cells were treated as shown for 1 h, and lysates were prepared. Twenty percent of each lysate was boiled in sample buffer to generate WCE, whereas the rest was precipitated with anti-GRIP1 antibody (αGRIP1 IP). Protein complexes were adsorbed on protein A/G Plus agarose beads, boiled in sample buffer, and separated by SDS-PAGE, along with WCE. GRIP1 and IRF9 were detected by immunoblotting. (E) GRIP1 overexpression in RAW 264.7 cells rescues ISG expression. RAW 264.7 cells (0.5×10^6) were transfected with 1 to 2 μg of pCDNA-GRIP1 (GRIP1) or empty vector (vec) using GenePORTER 3000 (Genlantis) as per the manufacturer's instructions. Cells were treated with IFN with or without Dex 24 h later for 2 h, and mRNA expression levels of IP10 and OASL1 were analyzed by qPCR, as described for Fig. 1.

precipitating the GRIP1-IRF9 complex and detecting GRIP1 at ISREs by ChIP.

Further corroborating our model, we were able to coimmunoprecipitate the GRIP1-IRF9 complex from RAW 264.7 cells with the same antibody, and in contrast to fibroblasts, Dex treatment resulted in the loss of IRF9 from GRIP1 (Fig. 7D), replicating the ISG expression pattern in BMMΦ and RAW 264.7 cells (Fig. 1A and data not shown) as well as ISGF3 and Pol II occupancy data in BMMΦ (Fig. 3). We reasoned that if a limited quantity of GRIP1 is at least in part responsible for the GC sensitivity of ISGF3-dependent gene transcription in MΦ, then exogenously provided GRIP1 may partially or fully restore ISG induction by IFN. Indeed, transiently introduced GRIP1 rescued IP10 and OASL1 expression in RAW 264.7 cells even in the presence of Dex (Fig. 7E). Interestingly, we were unable to generate RAW 264.7 sublines stably overexpressing GRIP1 under a selectable marker, as cells rapidly lost ectopic GRIP1 expression, perhaps indicating that tight regulation of the GRIP1 protein level is central to MΦ physiology.

DISCUSSION

Glucocorticoids are potent inhibitors of inflammatory and immune responses in both laboratory and clinical settings. The molecular mechanisms of their action are complex and involve multiple pathways; thus, a complete picture of inflammatory regulation by GCs remains elusive. For instance, GR directly activates transcription of several genes encoding established anti-inflammatory factors, including IκBα, annexin A1, IL-10, and GILZ (22). GR stimulates expression of the DUSP1 phosphatase, which dephosphorylates and inactivates mitogen-activated protein kinase (MAPK) p38 and Jun N-terminal protein kinase (JNK), which are essential for the induction/expression of numerous mediators of inflammation (1). GCs have also been shown to inhibit activating phosphorylation of TBK1, a kinase required for IRF3 activation in response to TLR3/4 signaling (43). Physical interactions between GR and T-cell receptors have recently been identified as a novel mode of GR-mediated immunosuppression in T cells (41). Finally, GR directly represses proinflammatory cytokine gene transcription through tethering to other transcription factors, such as NF-κB, AP1, CREB, T-bet, and NFAT (22). Despite their diversity, the above mechanisms all share a regulatory output: the attenuated expression of a host of cytokines, chemokines, and other mediators of inflammation. Here, we demonstrate that the type I IFN-initiated Jak/STAT signaling pathway itself is directly controlled by GR, revealing a previously unrecognized biological activity of GCs.

Although type I IFN signaling has been studied extensively, many questions remain. For example, the mediator component DRIP150 associates with ISGF3 and potentiates type I IFN-induced transcription (32); however, the functional relevance of the mediator complex as a whole in this context has not been resolved. STAT2 was shown to interact with the histone acetyltransferases CBP/p300 (9), but whether this recruitment results in sufficient chromatin remodeling to facilitate transcription is unclear. Our results suggest that the GRIP1 p160 family member is a direct, previously unrecognized coregulator of the ISGF3 complex required for the optimal expression of at least a subset of ISGs.

The p160 proteins, while best known as NR coactivators, are becoming increasingly appreciated as cofactors for multiple signaling pathways. GRIP1, in particular, has been shown to interact with and stimulate the activity of the myocyte enhancer factor-2C (Mef2C) and the IRF3 transcription complex

(15, 49). At least *in vitro*, GRIP1 binds several other IRF family members, including IRF1, IRF5, and IRF7 (8, 49) (unpublished data). SRC-1, another p160, potentiates the transcriptional activity of STAT3, STAT5a, STAT5b, and STAT6 through physical interactions between the transactivation domains of STATs and the PAS region of SRC-1 (26, 39). The broad role of p160 proteins as pleiotropic cofactors involved in such diverse transcriptional pathways raises questions regarding their specificity. Interestingly, although all three family members function as coactivators for NRs in overexpression studies and have been used in such assays interchangeably, a growing body of evidence points to the preferential recruitment by a given receptor of one p160 over another in a more physiological setting (59). Furthermore, despite the high degree of conservation of the PAS domains across the p160 family, GRIP1 and RAC3 did not substitute for SRC-1 in its regulation of STATs (39). Thus, it appears unlikely that SRC-1 or RAC3, which lack the domain equivalent to the IRF9-interacting GRIP1 RD, would be functionally redundant with GRIP1 with respect to ISGF3 coactivation.

If GRIP1 is the only p160 protein mediating the cross talk between GR and ISGF3, the reciprocal question is what promotes its selective recruitment to one regulator versus another? Clearly, the levels of the GRIP1 protein vary dramatically between different cell types, making certain cells, such as MΦ, uniquely receptive to signals that modulate its activity. Meanwhile, GRIP1-mediated pathways in other cell types may function relatively independently or lack a specific regulatory loop altogether. It is also likely that GRIP1 is differentially regulated posttranslationally, depending on the cell type and the nature of the signal. Indeed, the p160 family member SRC-3 displays a distinct phosphorylation fingerprint following treatment with 17β-estradiol compared to treatment with TNF-α, progesterone, or Dex (58, 61). Conceivably, IFN treatment of MΦ imparts posttranslational modifications to GRIP1 that preferentially direct it to the ISGF3 complex, whereas Dex triggers a different modification pattern that facilitates its binding to GR. Discerning such patterns will open up the possibility of signal manipulation, which should be of great therapeutic interest.

Our functional data illustrate that distinct ISGs are differentially affected by the loss of GRIP1, suggesting that the extent to which the ISGF3 complex relies on GRIP1 varies between the genes. This raises a question about the mechanistic role of GRIP1 in the context of IFN-activated genes. Coactivators, including the p160 family, stimulate transcription by recruiting histone-modifying enzymes, chromatin-remodeling complexes, and/or basal transcription machinery. Indeed, we show that IFN treatment modestly enhances acetylation of H3K9/14, which is partially blocked by Dex. Conceivably, GRIP1 enhances the recruitment of CBP/p300, its known interacting partner; however, as the basal levels of acetylation vary considerably from gene to gene, this mechanism may be important for only a subset of ISGs. The specific DNA sequence of and around the ISREs likely plays an essential role in determining whether and to what extent GRIP1 participates in regulation of a given ISG; in fact, nucleotide sequences appear to determine, in part, cofactor recruitment to many regulators, including the estrogen receptor (ER) (28). Remarkably, a single-base-pair substitution in a GRE leads to changes in GR structure, activity, and the composition of the associated coactivator complexes (44, 53).

In addition to serving as recruiters for secondary cofactors and the basal machinery, coactivators may also signal back to the cognate regulator by sterically stabilizing the regulatory complex itself. Indeed, loss of the cofactor MUC1 destabilizes ER and renders it susceptible to proteasomal degradation (56). Likewise, in the absence of cofactors, the yeast transcription factor Met4 dissociates from its ubiquitin ligase SCF^{Met30}, which leads to the proteasomal degradation of Met4 (13). In the case of NRs, p160s stabilize agonist in the ligand-binding pocket, thereby facilitating DNA binding by the receptor complexes. Here, we show that IFN-induced ISGF3 occupancy of its target promoters is dramatically reduced in Dex-treated MΦ, suggesting that perhaps GRIP1 stabilizes the complex in a given conformation, which may have a higher binding affinity or stability depending upon the specific DNA sequence. In this scenario, GRIP1 depletion by siRNA, Ad-Cre, or activated GR results in variable degrees of dissociation of the ISGF3 complex, effectively causing variable levels of Dex inhibition for different genes.

Identification of GRIP1 as a coactivator for IRF complexes appears somewhat paradoxical, given its role as a GR corepressor at the AP1 and NF-κB tethering GREs (50, 51). Because many ISGs are regulated by both ISGF3 and NF-κB, it is difficult to predict the transcriptional response to a pathogen that induces both the IFN-Jak/STAT pathway and the TLR pathway. In principle, the coactivator and corepressor functions of GRIP1 could operate concurrently, in which case the outcome may depend on the affinity of GRIP1 for either transcription factor, posttranslational modifications induced by the prevailing signal, or a combination thereof. The molecular switch for these functions is as yet unknown; however, deletion studies have shown that the GRIP1 activation domains (AD1/AD2) are inactive when GRIP1 is recruited as a corepressor at AP1 tethering GREs (50). Further mutational analysis and dissection of signal-specific posttranslational modifications may help to shed light on the molecular mechanisms of the GRIP1 coactivator/corepressor balance. The *in vivo* relevance of these functions to the equilibrium between the immunostimulatory and immunorepressive pathways requires mouse knock-in models in which GRIP1 will solely maintain one function or the other. Our results here suggest a unique role for GRIP1 as a fulcrum that controls the balance of many immunomodulatory pathways, and as such, understanding and exploiting its regulatory surfaces may provide new avenues of therapy for a multitude of immune-mediated diseases.

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